[3,17-23]. Thus, conserved viral proteins such as CA can be a promising antigen for vaccine-based CTL induction toward HIV control.

We previously showed vaccine-based control of a simian immunodeficiency virus mac239 (SIVmac239 [24]) challenge in a group of Burmese rhesus macaques possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [19,25]. $Gag_{206-216}$ (IINEEAADWDL) epitope-specific CTL responses play an important role in this control and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag (CA) with the cost of viral fitness [26]. However, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 [27], that has the same $Gag_{206-216}$ epitope sequence with SIVmac239; $Gag_{206-216}$-specific CTLs did not show responses against SIVsmE543-3 infection due to an aspartate (D)-to-glutamate (E) change, GagD205E, at Gag residue 205 [28].

Thus, the GagD205E substitution in SIVmac239 could result in viral escape from $Gag_{206-216}$-specific CTL recognition. However, in our previous analyses of *90-120-Ia*-positive animals eliciting $Gag_{206-216}$-specific CTL responses for one or two years postchallenge, we observed selection of GagL216S, but not GagD205E mutation in SIVmac239 infection, suggesting a possibility that the GagD205E substitution results in larger reduction of viral replicative ability than GagL216S. In the present study, we first constructed a mutant SIVmac239, SIVmac239Gag205E, with the GagD205E substitution and examined its replication ability in vitro. We found that this amino acid change in the CA NTD results in loss of viral fitness, which can be recovered by an additional amino acid change in the CA CTD. Further analyses presented in vitro and in vivo evidence for a structural constraint in the functional interaction between SIV CA NTD and CTD.

Results

Compensation for loss of viral fitness in SIVmac239Gag205E by additional GagV340M substitution

We first constructed a mutant SIVmac239 molecular clone DNA with a mutation of a D-to-E substitution at the 205th aa in Gag (CA NTD) to obtain the mutant virus, SIVmac239Gag205E (Figure 1). Analysis of viral replication kinetics on HSC-F, a macaque T cell line, revealed delayed peak of the mutant SIVmac239-Gag205E replication, indicating its lower replicative ability compared to the wild-type SIVmac239 (Figure 2).

We further followed up SIVmac239Gag205E replication on HSC-F cells and explored a possibility of viral reversion or additional mutations (Figure 3). No additional gag mutation became dominant on day 10 after SIVmac239Gag205E infection. Interestingly, however, in the second culture after passage of the first culture supernatants on day 10 into uninfected HSC-F cells, an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag (CA CTD), became dominant in two of four sets of experiments; SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. The GagD205E mutation remained dominant, and no other mutations were detected in the CA-coding region even in the second culture.

We then constructed a mutant SIVmac239 molecular clone DNA by introducing the GagV340M mutation into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E340M (Figure 1). This mutant SIV showed similar replication kinetics on HSC-F cells with the wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution (Figure 2). These results imply that SIV CA with Gag205D-340V or Gag205E-340M combination is functional whereas the CA with Gag205E-340V is less functional.

Possible interaction between Gag residues 205 and 340 in SIV CA hexamers

Recovery of viral fitness of SIVmac239Gag205E by the GagV340M substitution suggests a possibility of interaction between Gag residues 205 in the NTD and 340 in the CTD. Modeling of CA monomer structure, however, showed that the Gag 205th residue is located in the helix 4 of CA NTD, while the 340th is in the loop between helices 10 and 11 of CTD, which does not support a possibility of intramolecular contact between Gag residues 205 and 340 (data not shown).

CA molecules are known to form hexamer lattice in mature virions [29-33]. Modeling of CA hexamer structure revealed that the Gag 205th residue in the NTD is located in close proximity to the 340th in the CTD of the adjacent CA molecule (Figure 4). These observations support a possibility of intermolecular interaction between Gag residues 205 and 340 in CA hexamers.

In addition, the 312th residue in the loop between helices 8 and 9 of CTD is located in close proximity to the 205th in the NTD of the adjacent CA molecule. Because SIVmac239 and SIVsmE543-3 have different amino acids at this residue 312, alanine (A) in the former and proline (P) in the latter, we also constructed a mutant SIVmac239 molecular clone DNA by introducing the GagA312P mutation resulting in A-to-P substitution at the 312th aa in Gag into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E312P (Figure 1). Analysis of replication kinetics on HSC-F cells indicated recovery of viral fitness by the additional GagA312P substitution in SIVmac239Gag205E (Figure 2).

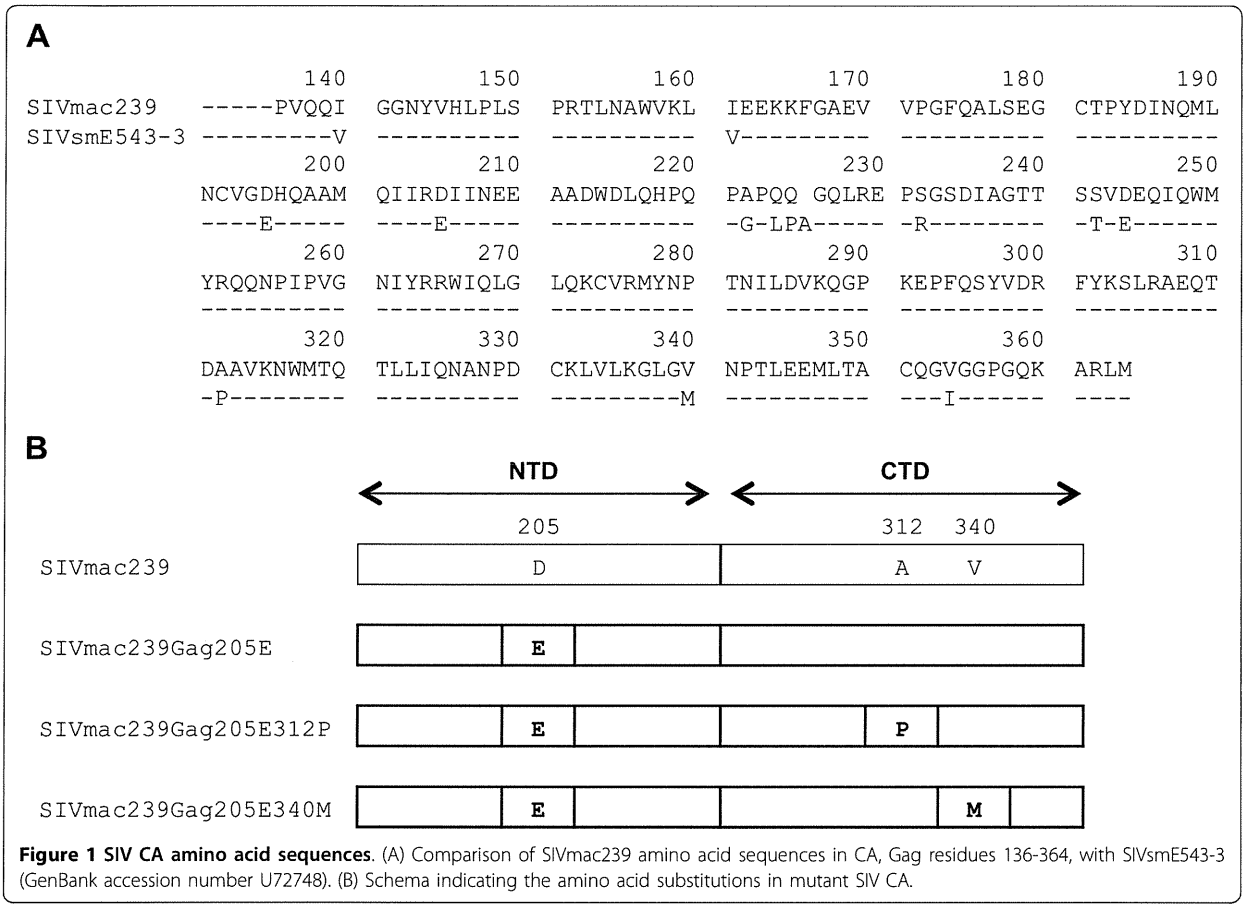

Figure 1 SIV CA amino acid sequences. (A) Comparison of SIVmac239 amino acid sequences in CA, Gag residues 136-364, with SIVsmE543-3 (GenBank accession number U72748). (B) Schema indicating the amino acid substitutions in mutant SIV CA.

Full recovery of viral fitness in SIVmac239Gag205E340M

We then focused on analyzing the possibility of functional interaction between Gag residues 205 in CA NTD and 312/340 in CA CTD. To confirm differences in viral fitness among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M, we compared their replicative ability by viral competition assay (Table 1). The competitions confirmed lower viral fitness of SIVmac239Gag205E compared to wild-type SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag340M. SIVmac239Gag205E312P showed lower viral fitness than SIVmac239, whereas replication ability of SIVmac239Gag205E340M was no less than the wild-type. These results indicate that the GagD205E substitution in SIVmac239 reduced viral fitness, which was recovered partially by an additional GagA312P and fully by an additional GagV340M substitution. The competition between SIVmac239 and SIVmac239Gag205E340M at the ratio of 1:1 resulted in selection of the latter, suggesting that SIV CA with Gag205E-340M combination observed in SIVsmE543-3 may be slightly more functional than that with Gag205D-340V in SIVmac239.

Inhibition of the early phase of SIVmac239Gag205E replication

We examined whether the GagD205E substitution affects the early or late phase of SIVmac239 replication. On LuSIV cells, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, SIVmac239Gag205E312P, or SIVmac239Gag205E340M, indicating suppression of the early phase of SIVmac239GagD205E replication (Figure 5). In contrast, we did not find a significant difference in viral production among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 6). These results indicate that the loss of viral fitness by the GagD205E substitution is mainly due to inhibition of the early phase of viral replication.

Loss of in vitro core stability in SIVmac239Gag205E

If the GagD205E substitution disturbs intermolecular CA interaction for hexamer formation, it may affect SIV core stability. To assess the core stability in vitro [34], concentrated viruses were separated into three fractions by ultracentrifugation under gradient sucrose

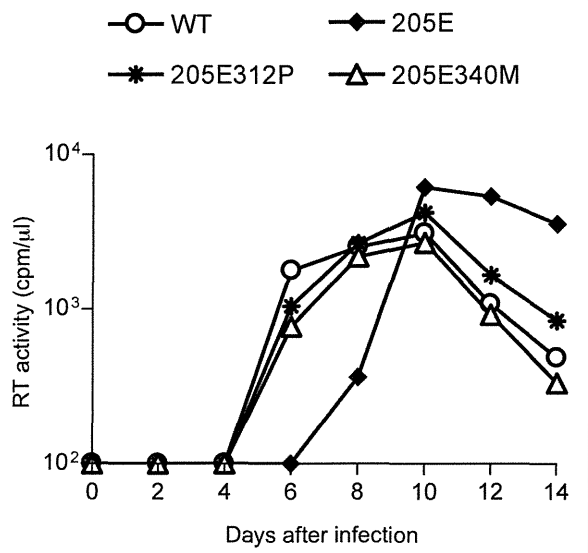

Figure 2 Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (WT, open circles), SIVmac239Gag205E (205E, closed diamonds), SIVmac239Gag205E312P (205E312P, asterisk), or SIVmac239Gag205E340M (205E340M, open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. A representative result from five sets of experiments is shown.

concentrations in the presence of Triton X-100 and each fraction was subjected to Western blot analysis to detect CA p27 proteins (Figure 7). In the absence of Triton X-100, CA proteins were detected in the bottom fraction, whereas those in the presence of 1% Triton X-100 were sensitive to the detergent and detected not in the bottom but only in the top fraction (data not shown). We compared the in vitro viral core stability between SIVmac239 and SIVmac239Gag205E in the presence of 0.6%, 0.9%, and 1.35% Triton X-100, respectively, and found a difference in the presence of 0.6% Triton X-100. Additional experiments revealed that SIVmac239Gag205E core was more sensitive to 0.6% Triton X-100 treatment than SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 7). These results suggest that viral core stability may be reduced by GagD205E substitution but can be recovered by additional GagA312P or GagV340M substitution.

Selection of GagD205E plus GagV340M mutations in a SIVmac239-infected macaque

The GagD205E substitution results in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition. Finally, we examined whether this substitution can be selected in the chronic phase of SIVmac239 infection in *90-120-Ia*-positive macaques eliciting $Gag_{206\text{-}216}$-specific CTL responses using plasma samples obtained in our previous experiments [35,36]. SIVmac239-infected *90-120-Ia*-positive macaques select the GagL216S mutation resulting in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition, but we found selection of both GagD205E and GagV340M mutations in viral genomes in one animal, R01-007 (Table 2). In this animal, GagD205E and GagV340M mutations were undetectable at week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant at week 123 was not detected at week 150. These results present in vivo evidence indicating functional interaction between the Gag 205th residue in NTD and the 340th in CTD of SIV CA.

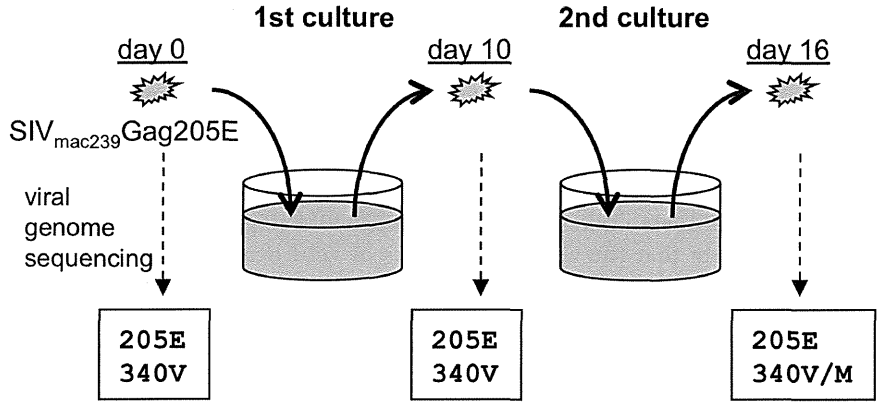

Figure 3 Passage of SIVmac239Gag205E culture supernatants. HSC-F cells were infected with SIVmac239Gag205E. The culture supernatant on day 10 was added to fresh HSC-F cells to start the second culture. Viral RNAs were extracted from the first culture supernatant on day 10 and the second culture supernatant on day 16 after the initial infection and subjected to sequence analyses. Dominant amino acid at the 340th residue remained V on day 10 in all cases but was M on day 16 in two of four sets of experiments (Gag340M was detectable on day 10 in these two sets of experiments). No other amino acid change was observed in the CA-coding region.

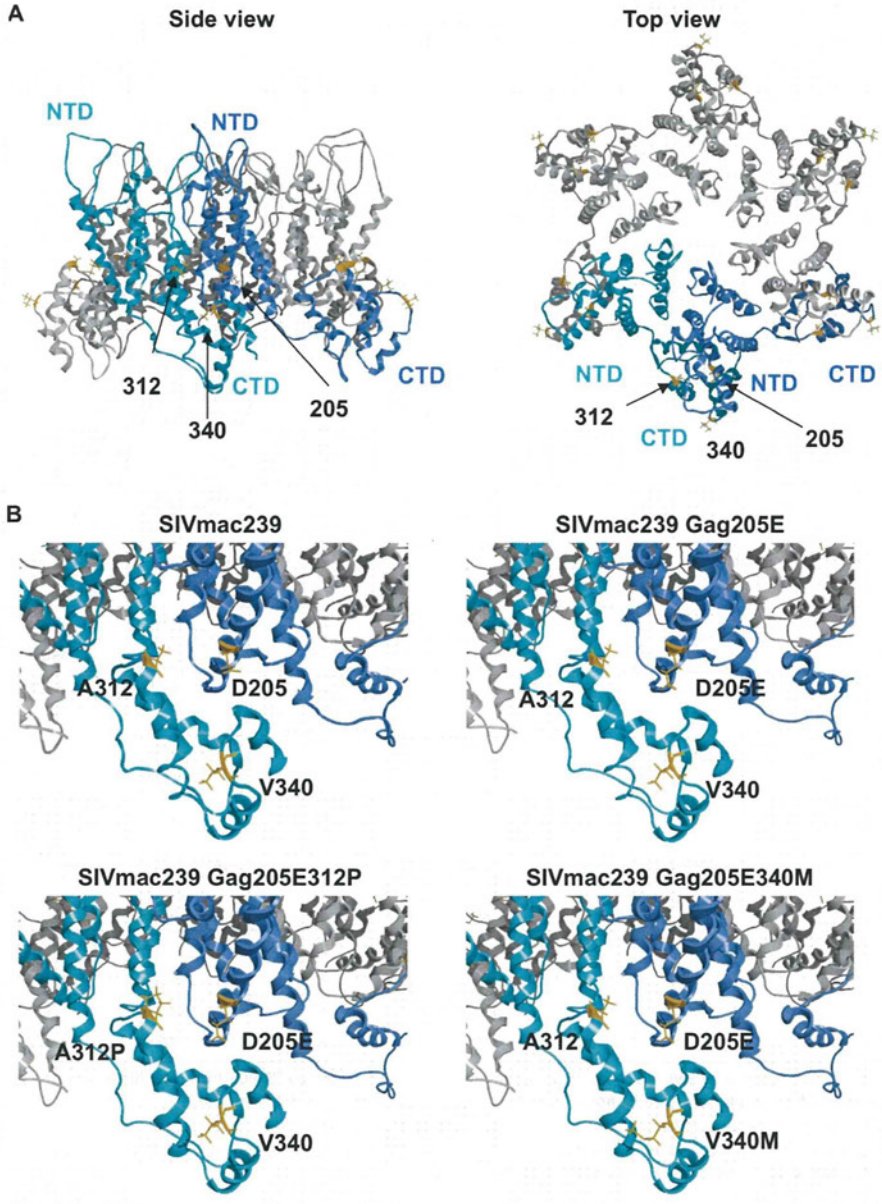

Figure 4 Structural models of SIVmac239 CA hexamer. The hexameric SIVmac239 CA models were constructed by homology-modeling using a crystal structure of the hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47[33]) as a modeling template. "MOE-Align" and "MOE-Homology" in MOE version 2008.1002 were used for the modeling. The side chains of the 205th, 312th, and 340th aa in Gag are shown as orange sticks. (A) Overall structure of SIVmac239 CA hexamer. (B) The hexameric structures near positions 205, 312, and 340 of wild-type and mutant SIVmac239 CAs.

Discussion

The Gag CA which is one of the most conserved proteins in HIV and SIV may be a promising immunogen for CTL-based AIDS vaccines. However, the limitations imposed on amino acid sequences in CA are not fully understood. In the present study, we found that the GagD205E substitution in SIVmac239 CA NTD reduces viral fitness, which is recovered by additional GagA312P or GagV340M substitution in the CTD. SIVmac239-Gag205E passaged in cell culture often resulted in selection of an additional GagV340M mutation. Furthermore, selection of Gag205E plus Gag340M mutations, but not Gag205E alone, was observed in a chronically SIVmac239-infected rhesus macaques. These results provide evidence indicating a functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD,

Table 1 Competition between SIV mutants[a]

SIVs in competition	Ratio of inoc. titers[b]	Exp. no.	Dominant aa sequences[c]			
			day 6		day 18	
SIVmac239 & SIVmac239Gag205E	4:1	#1	205D		205D	
		#2	205D		205D	
	1:1	#1	205D		205D	
		#2	205D		205D	
	1:4	#1	205D		205D	
		#2	205D		205D	
SIVmac239 & SIVmac239Gag205E312P	4:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:4	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
SIVmac239 & SIVmac239Gag205E340M	4:1	#1	205D	340V	205D	340V
		#2	205D	340V	205D	340V
	1:1	#1	205D/E	340V/M	205E	340M
		#2	205D/E	340V/M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
SIVmac239Gag205E & SIVmac239Gag205E312P	4:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:4	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
SIVmac239Gag205E & SIVmac239Gag205E340M	4:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M

[a]HSC-F cells were coinfected with two kinds of SIVs indicated. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants on days 6 and 18 postinfection and then sequenced. Results from two sets of experiments (Exp. #1 and #2) are shown.

[b]The ratio of the dose (RT activity) of the virus indicated at the top to that at the bottom at coinfection.

[c]Dominant amino acid sequences at the positions where mutations were included in the inoculums are shown. 205D/E, D and E were detected equally at the 205th aa in Gag; 340 V/M, V and M were detected equally at the 340th aa in Gag.

presenting a structural constraint for functional interaction between SIV CA NTD and CTD.

HIV and SIV Gag proteins are expressed as unprocessed polyproteins, which are assembled and incorporated into the virions. Concomitant with viral budding, incorporated Gag polyproteins are proteolytically cleaved by viral protease into processed proteins including MA (matrix), CA, and NC (nucleocapsid), participating in mature infectious virion formation [37,38]. Recent structural analyses [31-33,39-41] indicated that CA proteins form hexamer lattice in matured virions; in the mature CA core, the intermolecular NTD-NTD and NTD-CTD interfaces are involved in the formation of CA hexamers, while the intermolecular CTD-CTD interface connects neighboring hexamers. Our modeling analyses did not support a possibility of intramolecular interaction but indicated possible intermolecular interaction between Gag205 in CA NTD and Gag312/340 in CA CTD, which may affect CA hexamer formation during viral maturation. This is consistent with our results in Figure 5 indicating that the GagD205E substitution results in inhibition of the early phase of SIVmac239 replication, which can be overcome by additional GagA312P or GagV340M substitution. This possibility is supported also by our results on viral core stability in vitro, although it remains unclear how much extent the

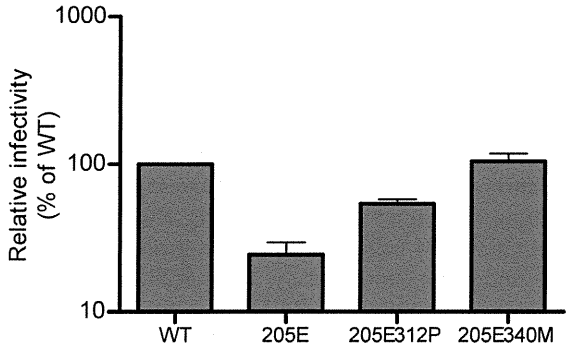

Figure 5 Mutant SIV infection. LuSIV cells were infected with SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340M (205E340M). Luciferase activity was measured 24 hr after infection. Relative infectivity is shown as the ratio (%) of the luciferase activity to that of SIVmac239 (WT). Mean values in three sets of experiments are shown.

core stability in vitro can reflect the one in vivo [42]. There has been no report suggesting the influence of the Gag 205 residue on SIV sensitivity to tripartite interaction motif 5α (TRIM5α). A previous report on HIV CA lattice [31,43] indicated a potential interaction between the helix 4 of NTD and the loop connecting helices 10 and 11 of CTD in the adjacent molecule. Our results suggest the possible involvement of Gag205 and Gag340 residues in this intermolecular NTD-CTD interaction in CA hexamers.

The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which looked to be compensated by GagV340M substitution (Figure 4). The modeling can draw a hydrophobic pocket between Gag205 and Gag340 residues in

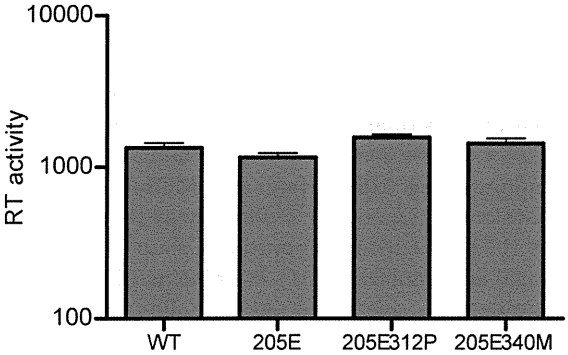

Figure 6 Mutant SIV production. COS-1 cells were transfected with molecular clone DNAs of SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340 M (205E340 M). RT activity of the culture supernatants two days after transfection was measured. Mean values in five sets of experiments are shown.

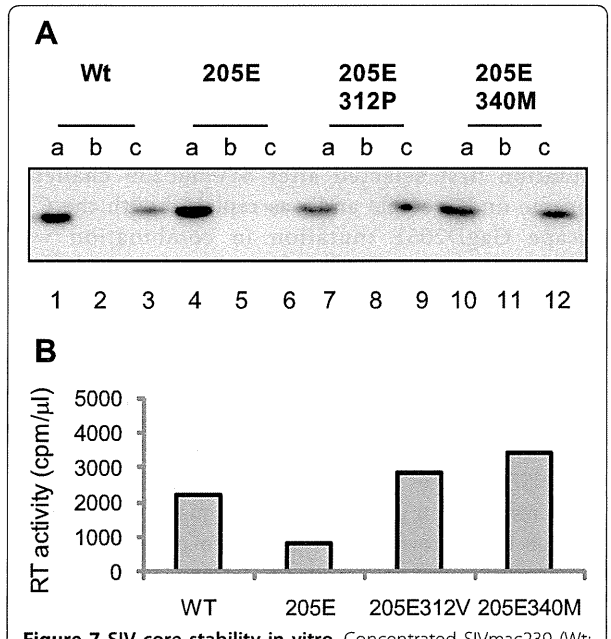

Figure 7 SIV core stability in vitro. Concentrated SIVmac239 (Wt; lanes 1-3), SIVmac239Gag205E (205E; lanes 4-6), SIVmac239Gag205E312P (205E312P; lanes 7-9), or SIVmac239Gag205E340M (205E340 M; lanes 10-12) was separated into three fractions (top [a], middle [b], and bottom [c]) by ultracentrifugation under gradient sucrose concentrations in the presence of 0.6% Triton X-100. Each fraction was subjected to Western blot analysis to detect SIV CA p27 proteins (A). A representative result from three sets of experiments is shown. The bottom (c) fractions were also subjected to RT assay (B).

SIVmac239Gag205E340M as well as SIVmac239, but not in SIVmac239Gag205E CA hexamers. Thus, this pocket may be a target candidate for anti-viral drugs.

Both GagL216S and GagD205E mutations can result in escape from $Gag_{206\text{-}216}$-specific CTL recognition [19,28], but the former is usually selected in SIVmac239-infected *90-120-Ia*-positive macaques probably

Table 2 Viral gag sequences in macaque R01-007 infected with SIVmac239[a]

Wks after challenge	Amino acid sequences[b]		
	at 205th	at 216th	at 340th
123	D	S	V
137	D (E)	S (L)	V (M)
150	E	L	M

[a]Viral RNAs were extracted from plasma obtained from a 90-120-Ia-positive macaque R01-007 at weeks 123, 137, and 150 after SIVmac239 challenge. Viral *gag* fragments were amplified by RT-PCR from viral RNAs and then sequenced. This animal showed efficient $Gag_{206\text{-}216}$-specific CTL responses and vaccine-based control of a SIVmac239 challenge with rapid selection of the GagL216S escape mutation (at week 5), but accumulated viral mutations in the chronic phase, leading to reappearance of plasma viremia around week 60 after challenge as described previously [19,35].

[b]Dominant amino acid sequences at the 205th, 216th, and 340th aa in Gag are shown. Parentheses indicate the sequences that are not dominant but detectable.

because the latter reduces viral fitness more severely than the former. In this study, we found selection of GagD205E plus GagV340M mutations in the chronic phase of SIVmac239 infection in a *90-120-Ia*-positive macaque. In this animal, the CTL escape GagL216S mutation first selected after SIVmac239 challenge became undetectable and was replaced with the CTL escape GagD205E mutation in combination with GagV340M in the chronic phase. This may imply that the GagD205E plus GagV340M mutations might be more advantageous than the GagL216S mutation for SIVmac239 replication in the presence of $Gag_{206-216}$-specific CTL pressure.

We observed the addition of GagV340M mutation but not a Gag205E-to-Gag205D reversion in SIVmac239-Gag205E passage. This may be due to difference in frequencies between purine-to-purine (guanine-to-adenine) change in the former and purine-to-pyrimidine (adenine-to-thymine) change in the latter. The appearance of additional GagV340M mutation in SIVmac239-Gag205E passaged in cell culture as well as the selection of GagD205E plus GagV340M mutations in an animal provides key evidence indicating functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD. The Gag is a promising candidate as a vaccine immunogen for CTL induction, because cumulative studies have indicated the efficacy of Gag-specific CTL responses against HIV and SIV infection [7,25,44,45]. However, viral mutational escape from CTL recognition is a major challenge for AIDS vaccine design. Thus, the information on the structural constraint presented in this study might be helpful for immunogen design in AIDS vaccine development.

Conclusions

Our results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. SIV CA with Gag205D-340V (observed in SIVmac239) or Gag205E-340M combination (observed in SIVsmE543-3) is functional whereas the CA with Gag205E-340V is less functional. Thus, the present study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing valuable information for immunogen design to limit viral escape options.

Methods

Analysis of mutant SIV replication

SIV molecular clone DNAs with gag mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 molecular clone DNA [24]. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs using Lipofectamine LTX PLUS (Invitrogen, Tokyo, Japan). Viral titers were measured by reverse transcription (RT) assay as described previously [46]. For analysis of viral replication kinetics, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) [47] were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. To examine viral infectivity, LuSIV cells, which are derived from CEMx174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, were cultured for 24 hr after viral infection and then lysed in a reporter lysis buffer (Promega Corp., Tokyo, Japan) for measurement of the luciferase activity in a luminometer (GloMax™ 96 Microplate Luminometer, Promega Corp.).

Viral competition assay

HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants harvested every other day were used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 post-coinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire gag region was amplified from the RNA by RT-PCR and sequenced to determine dominant sequences as described previously [19].

Molecular modeling of hexameric SIVmac239 CA

The crystal structures of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C[48]), HIV-1 CA CTD at a resolution of 1.70 Å (PDB code: 1A8O[5]), and hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47 [33]) were taken from the RCSB Protein Data Bank [49]. Three-dimensional (3-D) models of monomeric SIVmac239 CA were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) version 2008.1002 (Chemical Computing Group Inc., Quebec, Canada) as described [50,51]. We obtained 25 intermediate models per one homology modeling in MOE, and selected the 3-D models which were the intermediate models with best scores according to the generalized Born/volume integral methodology [52]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [53] combined with the generalized Born model of aqueous solvation implemented in MOE [54]. Physically unacceptable

local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE. The structures of hexameric SIVmac239 CA were generated from the monomeric structures by MOE on the basis of the assembly information of hexameric HIV-1 CA crystal structure [33].

Analysis of viral CA core stability in vitro

Detergent treatment of wild-type and mutant SIV particles was performed essentially as described previously [34]. Briefly, viruses from COS-1 cells transfected with viral molecular clone DNAs (normalized by RT activity) were concentrated by ultracentrifugation at 35,000 × rpm for 75 min at 4°C in a SW41 rotor (Beckman Instruments, Tokyo, Japan) through a cushion of 20% sucrose in phosphate buffered saline (PBS). The concentrated viral pellets were suspended in PBS. Sucrose step gradients were prepared in SW55 centrifuge tubes with the 2.0 ml layer of 60% sucrose on the bottom and 2.1 ml layer of 20% sucrose overlaid. Then, 0.1 ml of Triton X-100 in PBS and 0.5 ml of concentrated viruses were overlaid and ultracentrifuged at 35,000 × rpm for 60 min at 4°C in a SW55Ti rotor (Beckman Instruments). Three fractions (top [a], middle [b], and bottom [c]) of 1.1 ml each were collected from the top and subjected to Western blot analysis using plasma from a simian-human immunodeficiency virus 89.6PD-infected rhesus macaque [55] and RT assay.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, a grant-in-aid from the Japan Society for the Promotion of Science, grants-in-aid from the Ministry of Health, Labor, and Welfare, and a grant from Takeda Science Foundation in Japan. NI is a Research Fellow of the Japan Society for the Promotion of Science.

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Authors' contributions

NI and TM designed the study. NI, HT, and AR performed virological analyses in vitro. MY and HS performed structure modeling analyses. HY and MK examined viral genome sequences. NI and TM analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 23 August 2010 Accepted: 18 October 2010
Published: 18 October 2010

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doi:10.1186/1742-4690-7-90
Cite this article as: Inagaki *et al.*: A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins. *Retrovirology* 2010 **7**:90.

Biochemical and Biophysical Research Communications 408 (2011) 615–619

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

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ARTICLE INFO

Article history:
Received 12 April 2011
Available online 21 April 2011

Keywords:
AIDS vaccine
HIV
SIV
CTL
Immunodominance

ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

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1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

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doi:10.1016/j.bbrc.2011.04.071

$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

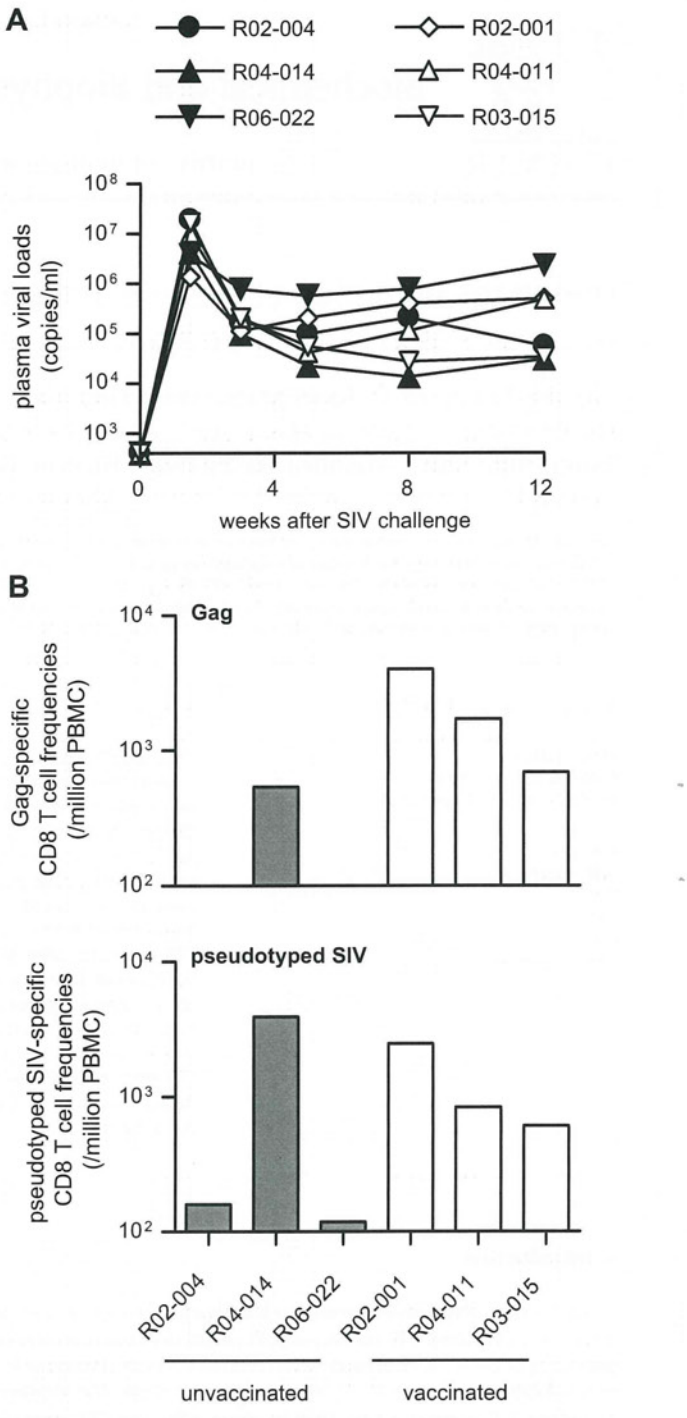

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

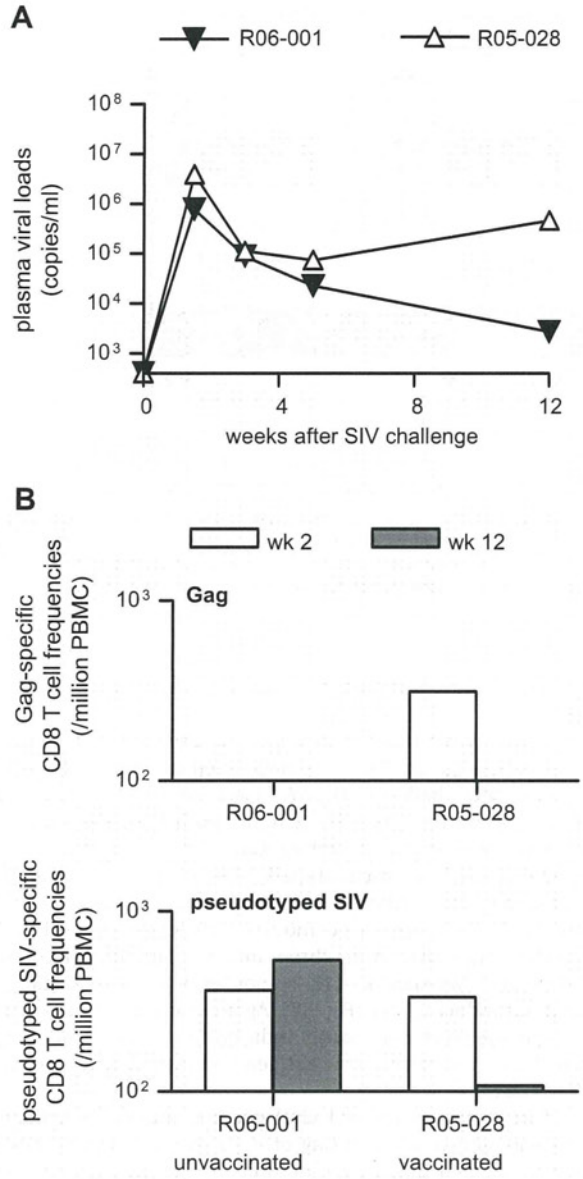

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

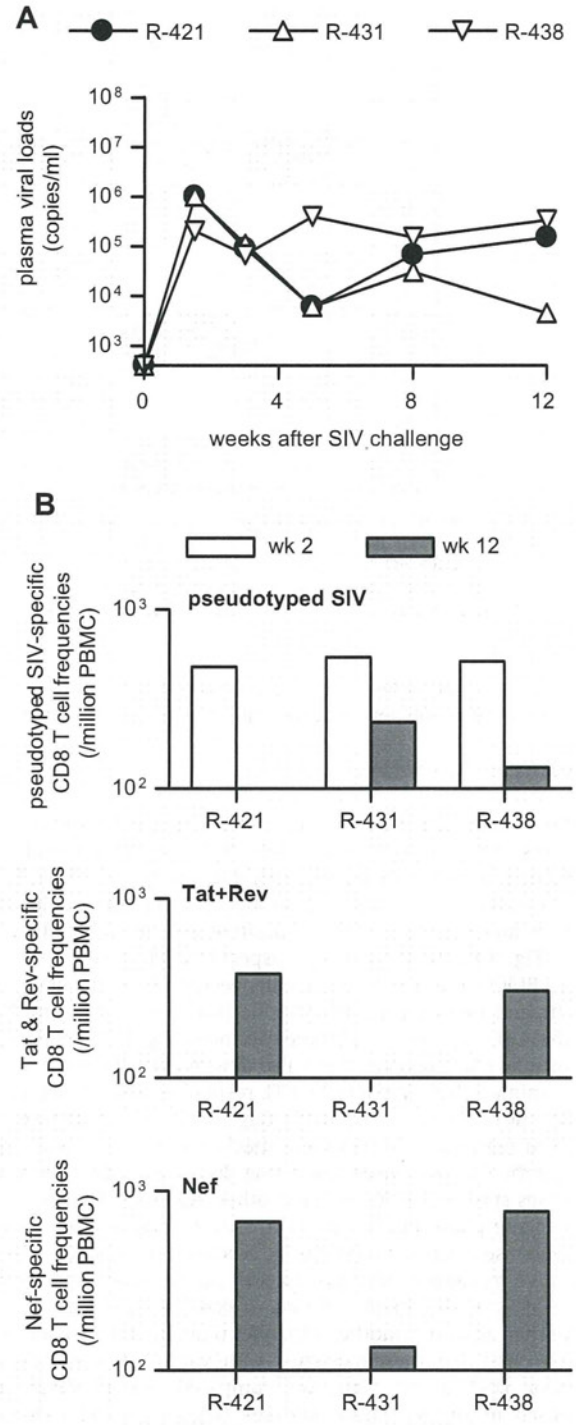

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

This work was supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, Grants-in-aid from the Ministry of Health, Labor, and Welfare, and a Grant from Takeda Science Foundation in Japan.

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Impact of Vaccination on Cytotoxic T Lymphocyte Immunodominance and Cooperation against Simian Immunodeficiency Virus Replication in Rhesus Macaques

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Cytotoxic T lymphocyte (CTL) responses play a central role in viral suppression in human immunodeficiency virus (HIV) infections. Prophylactic vaccination resulting in effective CTL responses after viral exposure would contribute to HIV control. It is important to know how CTL memory induction by vaccination affects postexposure CTL responses. We previously showed vaccine-based control of a simian immunodeficiency virus (SIV) challenge in a group of Burmese rhesus macaques sharing a major histocompatibility complex class I haplotype. $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CTL responses were responsible for this control. In the present study, we show the impact of individual epitope-specific CTL induction by prophylactic vaccination on postexposure CTL responses. In the acute phase after SIV challenge, dominant $Gag_{206-216}$-specific CTL responses with delayed, naive-derived $Gag_{241-249}$-specific CTL induction were observed in $Gag_{206-216}$ epitope-vaccinated animals with prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory, and vice versa in $Gag_{241-249}$ epitope-vaccinated animals with single $Gag_{241-249}$ epitope-specific CTL induction. Animals with $Gag_{206-216}$-specific CTL induction by vaccination selected for a $Gag_{206-216}$-specific CTL escape mutation by week 5 and showed significantly less decline of plasma viral loads from week 3 to week 5 than in $Gag_{241-249}$ epitope-vaccinated animals without escape mutations. Our results present evidence indicating significant influence of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses, which affects virus control. These findings provide great insights into antigen design for CTL-inducing AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces chronic, persistent viral replication leading to AIDS onset in humans. Virus-specific cytotoxic T lymphocyte (CTL) responses play a central role in the resolution of acute peak viremia (3, 4, 13, 22, 28) but mostly fail to contain viral replication in the natural course of HIV infection. Vaccination resulting in more effective CTL responses after viral exposure than in natural HIV infections would contribute to HIV control (30, 33). CTL memory induction by prophylactic vaccination may lead to efficient secondary CTL responses, but naive-derived primary CTL responses specific for viral nonvaccine antigens can also be induced after viral exposure. It is important to know how CTL memory induction by vaccination affects these postexposure CTL responses.

Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression (5, 14, 31, 34). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in this viral control (1, 8, 23, 24). Indian rhesus macaques possessing certain major histocompatibility complex class I (MHC-I) alleles, such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*, tend to show simian immunodeficiency virus (SIV) control (19, 25, 36). This implies possible HIV control by induction of particular effective CTL responses (2, 7, 12, 16, 27).

Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated the possibility of reduction in post-challenge viral loads (6, 15, 17, 21, 35). We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (20). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (21). Animals possessing *90-120-Ia* dominantly elicited Mamu-A1*043:01 (GenBank accession number AB444869)-restricted $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and Mamu-A1*065:01 (AB444921)-restricted $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses after SIV challenge and selected for viral *gag* mutations, GagL216S (leading to a leucine [L]-to-serine [S] substitution at amino acid [aa] 216 in Gag) and GagD244E (aspartic acid [D]-to-glutamic acid [E] at aa 244), resulting in escape from CTL recognition with viral fitness costs in the chronic phase (9, 26). Vaccinees possessing *90-120-Ia* failed to control a challenge with a mutant SIV carrying these two CTL escape mutations, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses play a crucial role in the vaccine-based control of wild-type SIVmac239 replication

Received 5 September 2011 **Accepted** 31 October 2011
Published ahead of print 9 November 2011
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doi:10.1128/JVI.06226-11

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TABLE 1 Animals analyzed in this study

Group	No. of animals	Vaccination[a]	SIV-specific CTL response postboost
I	6	None	None
II	5	Gag (pCMV-SHIVdEN DNA prime, SeV-Gag boost)	Gag-specific CTL
III	6	$Gag_{241-249}$-specific (pGag$_{236-250}$-EGFP-N1 DNA prime, SeV-Gag$_{236-250}$-EGFP boost)	$Gag_{241-249}$-specific CTL
IV	5	$Gag_{206-216}$-specific (pGag$_{202-216}$-EGFP-N1 DNA prime, SeV-Gag$_{202-216}$-EGFP boost)	$Gag_{206-216}$-specific CTL

[a] All animals were challenged with SIVmac239.

(10). Furthermore, in an SIVmac239 challenge experiment with *90-120-Ia*-positive rhesus macaques that received a prophylactic vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), this single-epitope vaccination resulted in control of SIVmac239 replication with dominant induction of $Gag_{241-249}$-specific CTL responses in the acute phase postchallenge (32).

Thus, it is hypothesized that induction of single $Gag_{206-216}$ or $Gag_{241-249}$ epitope-specific CTL responses by vaccination may result in different patterns of CTL immunodominance and viral replication after SIV challenge. In the present study, we analyzed the impact of prophylactic vaccination inducing single $Gag_{206-216}$ epitope-specific CTL responses on SIV control in *90-120-Ia*-positive macaques and compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. This analysis revealed differences in CTL responses and patterns of viral control after SIV challenge between these vaccinated groups, indicating significant effects of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses.

MATERIALS AND METHODS

Animal experiments. Animal experiments were conducted through the Cooperative Research Program at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. All animals were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases.

Five Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia* (26) (group IV) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206-216}$-specific CTL responses followed by an SIVmac239 challenge and were compared with three groups (I, II, and III) of *90-120-Ia*-positive animals reported previously (10, 32) (Table 1). Group I animals ($n = 6$) received no vaccination, while group II animals ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting Gag-specific CTL responses. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from a simian/human immunodeficiency virus ($SHIV_{MD14YE}$) molecular clone DNA with *env* and *nef* deleted (29) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239–HIV-1 chimeric Vpr; and HIV-1 Tat and Rev (21). In group II animals, CTL responses were undetectable after DNA prime but Gag-specific CTL responses became detectable after SeV-Gag boost. Group III animals ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{241-249}$-specific CTL responses. A pGag$_{236-250}$-EGFP-N1 DNA and an SeV-Gag$_{236-250}$-EGFP vector, both expressing an SIVmac239 $Gag_{236-250}$ (IAGTTSSVDEQ IQWM)-EGFP fusion protein, were used for the group III vaccination. After the SeV-Gag$_{236-250}$-EGFP boost, group III animals induced $Gag_{241-249}$-specific CTL responses; the animals showed no $Gag_{236-250}$-specific $CD4^+$ T-cell responses but elicited SeV/EGFP-specific $CD4^+$ T-cell responses (32). For the group IV vaccination, A pGag$_{202-216}$-EGFP-N1 DNA and an SeV-Gag$_{202-216}$-EGFP vector, both expressing an SIVmac239 $Gag_{202-216}$ (IIRDIINEEAADWDL)-EGFP fusion protein, were used (Fig. 1). Approximately 3 months after the boost, all animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (11). In our previous study (32), the unvaccinated and the control-vaccinated

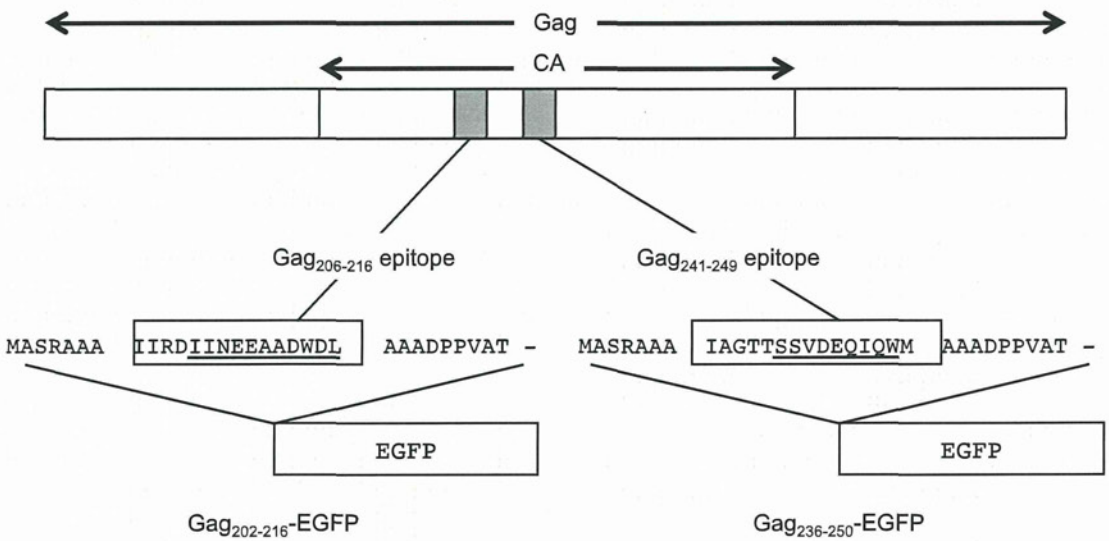

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

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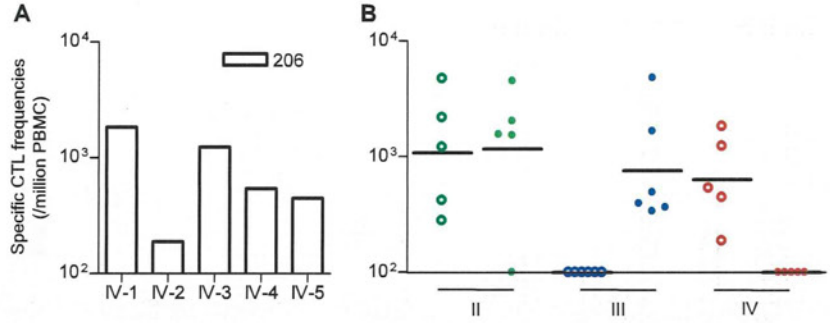

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (closed circles) $CD8^+$ T-cell frequencies 1 week after boost in group II (green), III (blue), and IV (red) macaques. The bars indicate the geometric mean of each group. No animal showed detectable Gag-specific CTL responses before the boost.

animals receiving a DNA and an SeV expressing EGFP showed no significant differences in viral loads after SIV challenge.

Analysis of antigen-specific CTL responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (21). Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papioimmortalized B-lymphoblastoid cell lines pulsed with 1 μM SIVmac239 $Gag_{206-216}$ (IINEEAADWDL), $Gag_{241-249}$ (SSVDEQIQW), or $Gag_{367-381}$ (ALKEALAPVPIPFAA) peptide for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-specific stimulation. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ (Biolegend, San Diego, CA) monoclonal antibodies. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

Sequencing of the viral genome. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Fragments corresponding to nucleotides from 1231 to 2958 (containing the entire *gag* region) in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested reverse transcription (RT)-PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using R software (R Development Core Team). Differences in geometric means of plasma viral loads were examined by one-way analysis of variance (ANOVA) and Tukey-Kramer's multiple-comparison test. Plasma viral loads at week 3 were examined for differences between group III and groups II and IV by analysis of covariance (ANCOVA) with week 5 viral loads as a covariate.

RESULTS

CTL responses after prophylactic vaccination. We previously reported the efficacy of vaccination eliciting whole Gag-specific or single $Gag_{241-249}$ epitope-specific CTL memory against SIVmac239 challenge (10, 32). In the present study, we examined the efficacy of prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory against SIVmac239 challenge and compared the results with those of the previous experiments.

Five Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{206-216}$ epitope-specific CTL responses. A plasmid DNA (p$Gag_{202-216}$-EGFP-N1) and an SeV (SeV-$Gag_{202-216}$-EGFP) vector, both expressing an SIVmac239 $Gag_{202-216}$-EGFP fusion pro-

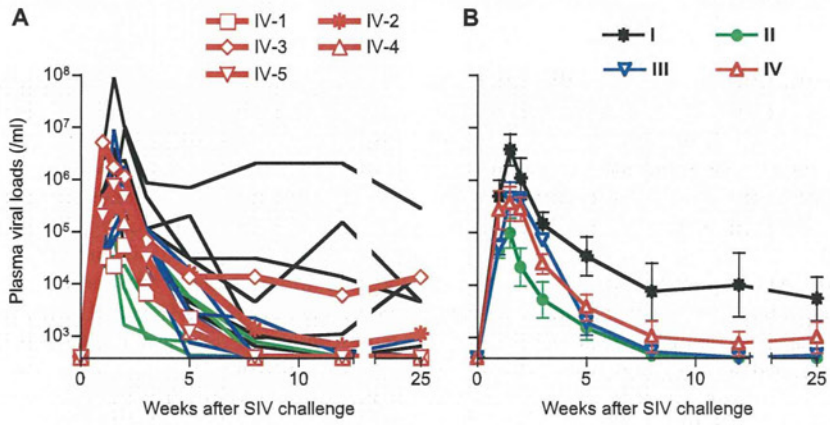

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) after challenge. (B) Changes in geometric means of plasma viral loads after challenge. Groups II and III (but not group IV) showed significantly lower set point viral loads than group I ($P = 0.0390$ between groups I and II, $P = 0.0404$ between groups I and III, and $P > 0.05$ between groups I and IV at week 25 by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

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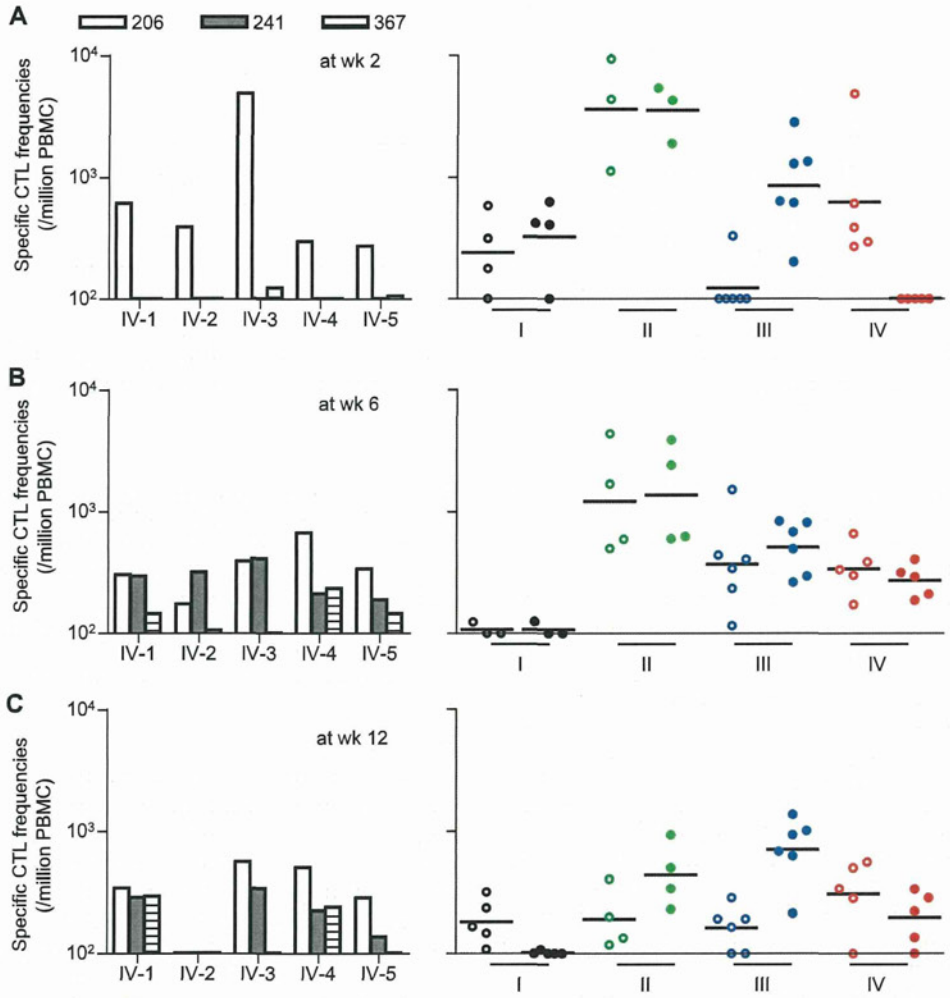

FIG 4 $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206\text{-}216}$-specific (open boxes), $Gag_{241\text{-}249}$-specific (closed boxes), and $Gag_{367\text{-}381}$-specific (striped boxes) $CD8^+$ T-cell frequencies in group IV macaques are shown. On the right, $Gag_{206\text{-}216}$-specific (open circles) and $Gag_{241\text{-}249}$-specific (closed circles) $CD8^+$ T-cell frequencies in group I (black), II (green), III (blue), and IV (red) macaques are shown. The bars indicate the geometric mean of each group. Samples from macaques I-1, I-6, II-1, and II-3 at week 2; macaques I-1, I-2, I-6, and II-5 at week 6; and macaques I-1 and II-5 at week 12 were unavailable for this analysis. Statistical analyses among four groups at week 12 revealed significant differences in $Gag_{241\text{-}249}$-specific CTL levels (I and III, $P < 0.0001$; I and II, and III and IV, $P < 0.01$; I and IV, II and III, and II and IV, $P > 0.05$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test) but not in $Gag_{206\text{-}216}$-specific CTL levels ($P > 0.05$ by one-way ANOVA).

tein, were used for the vaccination (Fig. 1). We confirmed $Gag_{206\text{-}216}$-specific CTL responses 1 week after SeV-$Gag_{202\text{-}216}$-EGFP boost in all five animals (Fig. 2A). As expected, no $Gag_{241\text{-}249}$-specific CTL responses were detected in these animals. No $Gag_{202\text{-}216}$-specific $CD4^+$ T-cell responses were detected in the animals except for one (IV-5) showing marginal levels of responses (data not shown).

Plasma viral loads after SIV challenge. We compared these five animals (referred to as group IV) with other groups (I, II, and III) of *90-120-Ia*-positive macaques reported previously (Table 1). Group I animals ($n = 6$) received no vaccination, group II ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting whole Gag-specific CTL responses, and group III ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were detectable after SeV-Gag boost in four of five group II animals except for one animal (II-3), in which $Gag_{206\text{-}216}$-specific, but not $Gag_{241\text{-}249}$-specific, CTL responses were detected. In all group III animals, $Gag_{241\text{-}249}$-specific CTL responses were confirmed, while no $Gag_{206\text{-}216}$-specific CTL responses were detected after SeV-$Gag_{236\text{-}250}$-EGFP boost (Fig. 2B).

After SIVmac239 challenge, all animals were infected and showed plasma viremia during the acute phase. Plasma viremia was maintained in five of six unvaccinated animals in group I but became undetectable in one animal (I-2) at week 12. In contrast, all animals in groups II and III contained SIV replication with significantly reduced plasma viral loads compared to group I at the set point. In group IV, however, vaccine efficacy was not so clear; while three out of five animals contained SIV replication, the remaining two (IV-2 and IV-3) failed to control viral replication with persistent plasma viremia (Fig. 3).

Gag-specific CTL responses after SIV challenge. We then measured $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge by detection of peptide-

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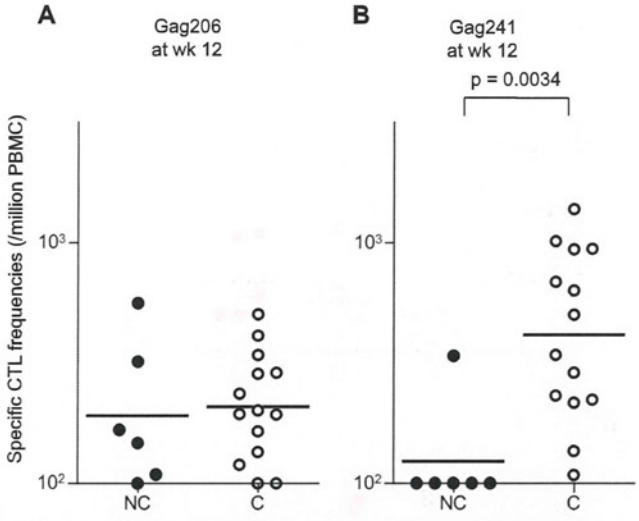

FIG 5 Comparison of $Gag_{206\text{-}216}$-specific or $Gag_{241\text{-}249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206\text{-}216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241\text{-}249}$-specific $CD8^+$ T-cell frequencies in noncontrollers and controllers. $Gag_{241\text{-}249}$-specific CTL levels in controllers were significantly higher than those in noncontrollers ($P = 0.0034$ by Mann-Whitney test). The bars indicate the geometric mean of each group. Data on a noncontroller (I-1) and a controller (II-5) were unavailable.

specific IFN-γ induction. At week 2 (Fig. 4A), most animals in groups I and II elicited both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses, whereas group III animals induced $Gag_{241\text{-}249}$-specific CTL responses dominantly. Remarkably, all animals in group IV showed efficient $Gag_{206\text{-}216}$-specific CTL responses without detectable $Gag_{241\text{-}249}$-specific CTL responses at week 2. These results indicate dominant $Gag_{206\text{-}216}$-specific CTL responses with delayed induction of $Gag_{241\text{-}249}$-specific CTL responses postchallenge in group IV animals with prophylactic $Gag_{206\text{-}216}$-specific CTL induction, and vice versa in group III animals.

At week 6 (Fig. 4B), efficient $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were observed in all vaccinated animals in groups II, III, and IV, but not in group I. $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were induced equivalently even in groups III and IV. We also examined subdominant $Gag_{367\text{-}381}$ epitope-specific CTL responses, which were undetectable at week 2 but became detectable at week 6 in most group IV animals (Fig. 4, graphs on left). At week 12 (Fig. 4C), however, different CTL immunodominance patterns were observed among the groups. $Gag_{241\text{-}249}$-specific CTL levels were higher than $Gag_{206\text{-}216}$-specific levels in groups II and III but were reduced in groups I and IV. Interestingly, comparison between the animals with persistent viremia (referred to as noncontrollers) and those controlling SIV replication (referred to as controllers) revealed significant differences in $Gag_{241\text{-}249}$-specific CTL levels, but not in $Gag_{206\text{-}216}$-specific levels, at week 12 ($P = 0.0034$ by Mann-Whitney test) (Fig. 5).

Selection of a CTL escape mutation. Next, we examined viral genome *gag* sequences at weeks 5 and 12 after challenge to determine whether CTL escape mutations were selected in these animals (Table 2). At week 5, a mutation leading to an L-to-S substitution at the 216th residue in Gag (L216S) was selected in all the group II animals. This GagL216S change results in escape from $Gag_{206\text{-}216}$-specific CTL recognition, as described previously (21). All the group IV animals with $Gag_{206\text{-}216}$-specific CTL induction also showed rapid selection of this CTL escape mutation at week 5. Analysis at week 3 found the GagL216S mutation dominant in two (II-2 and II-5) group II and two (IV-1 and IV-3) group IV animals (data not shown). However, animals in group III showed no *gag* mutations at week 5, except for one animal (III-5) selecting a mutation leading to an L-to-F substitution at the 216th residue. Later, at week 12, the $Gag_{206\text{-}216}$-specific CTL escape mutation, GagL216S, was selected even in group III animals. No animals showed mutations around the $Gag_{241\text{-}249}$ epitope-coding region even at week 12. These results indicate that selection of this $Gag_{206\text{-}216}$-specific CTL escape mutation may be accelerated by prophylactic vaccination inducing $Gag_{206\text{-}216}$-specific CTL responses. On the other hand, in group III animals with single $Gag_{241\text{-}249}$ epitope-specific CTL induction, selection of a $Gag_{206\text{-}216}$-specific CTL escape mutation was delayed but was observed before selection of a $Gag_{241\text{-}249}$-specific CTL escape mutation, suggesting strong selective pressure by delayed $Gag_{206\text{-}216}$-specific CTL responses after SIV challenge.

In order to see the effect of rapid selection of the $Gag_{206\text{-}216}$-specific CTL escape mutation on SIV control, we compared plasma viral loads at weeks 3 and 5 between groups II and IV (referred to as group II+IV) with rapid selection of the GagL216S

TABLE 2 Selection of a CTL escape mutation

Group	Macaque ID	Amino acid change for Gag residues[b]: 206–216		241–249	
		Wk 5	Wk 12	Wk 5	Wk 12
I	I-1	None	ND	None	ND
	I-2[a]	None	L216S	None	None
	I-3	None	L216S	None	None
	I-4	None	None	None	None
	I-5	None	None	None	None
	I-6	None	None	None	None
II	II-1[a]	L216S	ND	None	ND
	II-2[a]	L216S	ND	None	ND
	II-3[a]	L216S	ND	None	ND
	II-4[a]	L216S	ND	None	ND
	II-5[a]	L216S	ND	None	ND
III	III-1[a]	None	L216S	None	None
	III-2[a]	None	L216S	None	None
	III-3[a]	None	NA	None	NA
	III-4[a]	None	NA	None	NA
	III-5[a]	L216F	L216S	None	None
	III-6[a]	None	L216S	None	None
IV	IV-1[a]	L216S	L216S	None	None
	IV-2	L216S	L216S	None	None
	IV-3	L216S	L216S	None	None
	IV-4[a]	L216S	L216S	None	None
	IV-5[a]	L216S	NA	None	NA

[a] Animals that controlled SIV replication at week 12 (controllers).
[b] Plasma viral gag genome mutations were examined at weeks 5 and 12. Amino acid substitutions in $Gag_{206\text{-}216}$ and $Gag_{241\text{-}249}$ epitope regions are shown. L216S results in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition. It remains undetermined whether L216F results in CTL escape. ND, not determined; NA, not determined because Gag fragments were unable to be amplified from plasma RNA.

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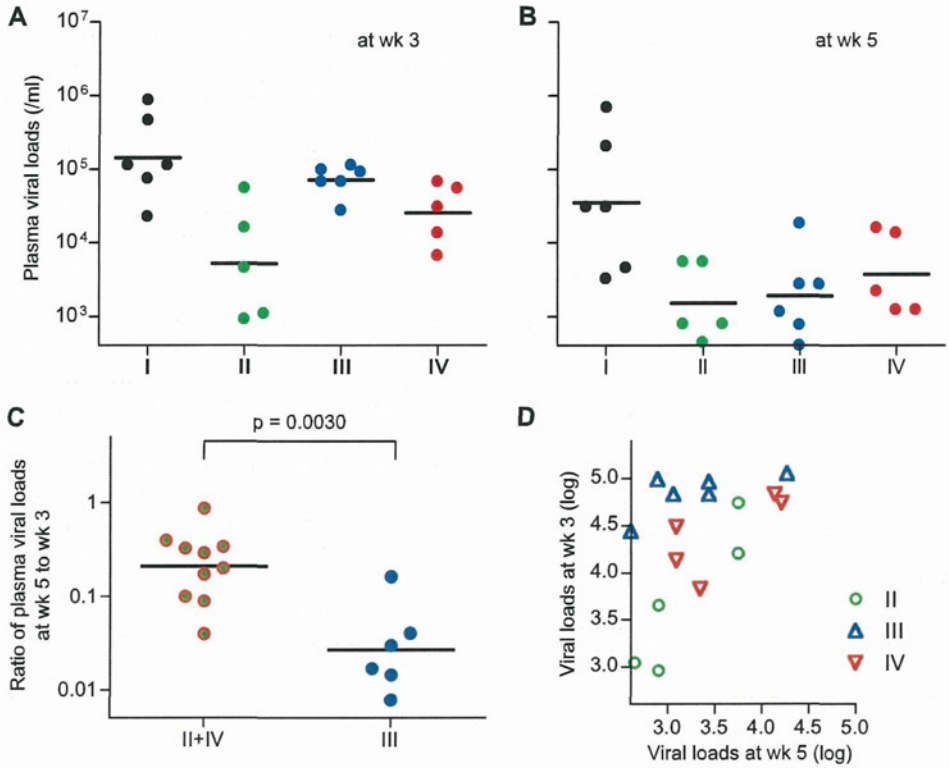

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals and group III animals. The ratios in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test). The bars indicate the geometric mean of each group. (D) Scatter plots between plasma viral loads at weeks 3 and 5 in group II, III, and IV animals.

mutation and group III without the mutation at week 5 (Fig. 6). Ratios of plasma viral loads at week 5 to week 3 in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test) (Fig. 6C). To confirm this result, we examined the difference in week 3 viral loads between groups III and II+IV by ANCOVA, with week 5 viral loads as a covariate. This analysis revealed that week 3 viral loads controlled for by week 5 viral loads were significantly higher in group III than those in group II+IV (Fig. 6D and Table 3); i.e., the decline in viral loads from week 3 to week 5 was significantly sharper in group III than in group II+IV, possibly reflecting viral escape from suppressive pressure by $Gag_{206\text{-}216}$-specific CTL responses in the latter group during this period (from week 3 to week 5).

DISCUSSION

In the present study, we analyzed the impact of vaccination inducing single $Gag_{206\text{-}216}$ epitope-specific CTL memory on postchallenge CTL responses and SIV control in *90-120-Ia*-positive macaques and then compared the results with those of vaccination inducing single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Our results indicate that these prophylactic vaccinations result in different patterns of $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL immunodominance and cooperation after SIVmac239 challenge.

Unvaccinated *90-120-Ia*-positive macaques (group I) showed both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIV challenge. In group IV animals with prophylactic induc-

TABLE 3 ANCOVA on week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV

ANOVA	Parameter	SS[a]	df[b]	MS[c]	*F*	*P* value
Homogeneity of slopes of regression	Group × slope	0.304	1	0.304	2.099	0.173
	Residual	1.735	12	0.145		
	Total	2.038	13	0.157		
Difference in week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV	Effect and group	1.106	1	1.106	7.052	0.020
	Residual	2.038	13	0.157		
	Total	3.144	14	0.225		

[a] SS, sum of squares.
[b] df, degrees of freedom.
[c] MS, mean squares.

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